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Disease resistance for different livestock species

Till now, we have described the genetics of the livestock population generally in terms of classical breeding and its molecular aspects. In this current chapter, we shall deal with progress in disease resistance for the livestock population species-wise and describe progress globally.

Disease resistance for cattle and buffalo

Cattle and buffalo form the major backbone for the dairy industry in any country. Efforts are in progress to develop a disease-resistant stock of dairy animals.

A school of thought implies that disease resistance for cattle depends on several factors including the adaptability, protective ability, and immune and healing mechanisms of cattle.

In a field-level study, it was observed that buffaloes were quite resistant to a majority of diseases, specifically systemic infectious diseases, compared with resistance evident in goats, sheep, and cattle. One of the most interesting observations is that a negligible number of buffaloes under study had systemic infections in terms of febrile reactions or any type of contagious diseases, especially foot-and-mouth disease in winter. Attempts were made for direct selection of cattle against brucellosis. An increase in natural resistance to brucellosis in calves, from 20% to 59% after breeding cows to a naturally resistant bull, has been reported. The natural resistance-associated macrophage protein 1 (NRAMP1) gene has been linked with resistance to brucellosis, tuberculosis, and salmonellosis. *Major histocompatibility complex (MHC)* genes are linked to specific immunological responses. A region on chromosome 1 was associated with infectious keratoconjunctivitis (pinkeye) in cattle, which is heritable. *Bos indicus* breeds were observed to have better resistance to tick infestation and tick-borne diseases, as revealed through higher hemolytic complement activity.

Disease resistance for mastitis

Mastitis is one of the most costly diseases for the dairy industry and has a huge economic impact. Mammary gland infections cost the US dairy industry approximately $2 billion dollars annually and have a similar impact in Europe. The major problem in controlling mastitis is its multietiology. Several breeding strategies have been employed along with molecular parameters, but none have been proved 100% effective.

An effort was employed that produced transgenic cows resistant to mastitis. A lysostaphin gene insert was used that expresses lysostaphin in the mammary epithelium and secretes the antimicrobial peptide into milk. Thus the transgenic cattle secreting lysostaphin kill the major pathogen (*Staphylococcus aureus*) in a dose-dependent manner. Similar attempts are in progress to develop protection from mastitis for dairy cattle through the introduction of other genes. However, it should be noted that the milk's nutritional and manufacturing properties are not altered. Multicistronic constructs may be required to achieve the goals, as will other strategies possibly involving RNA interference (RNAi) and gene-targeting technology. Thus a new avenue has developed for the production of disease-resistant animals through a transgenic approach.

Similar transgenic approaches can be equally applicable using other genes effective in creating resistance to mastitis. A list is available in one of our earlier review papers.

Genes identified for mastitis resistance

◆ Beta-defensin
◆ Lactoferrin
◆ CD14
◆ Interleukin-8 receptor
◆ Toll-like receptors (TLRs) TLR2 and TLR4
◆ Lysozyme
◆ MHC
◆ Cathelicidin
◆ Butyrophilin
◆ Interferon

◆ Lysostaphin*
◆ Molecule possessing ankyrin repeats induced by lipopolysaccharides
◆ Lanthionine−containing antimicrobial peptide
◆ Signal transducer and activator of transcription 3
◆ Nuclear factor-κβ (NF-κβ)
◆ Polymeric immunoglobulin gene (pIgR)
◆ Tumor necrosis factor (TNF), or cachectin

Other genes reportedly responsible for mastitis resistance were BNBD5, IgJ, MIP-3, MnSOD, AHCY, WIP, PRKDC, HNRPU, and OSTF1.

Experimental evidence has also proved the role of the peptide coded by these genes in preventing mastitis. Mastitis increased mammary mRNA of β-defensin 5, TLR2, and TLR4 in cattle as evidenced by real-time polymerase chain reaction (RT-PCR). In situ hybridization revealed that BNBD-5 is expressed predominantly in the mammary epithelial cells of the infected mammary gland. The role of MHC in susceptibility or resistance to intramammary infection has been documented.

Expression profile of genes associated with mastitis in dairy cattle

The differential mRNA expression profile was studied from the milk cells of healthy cows and cows with clinical mastitis. The genes under study were IL2, IL4, IL6, IL8, IL10, IFN-γ, and TNF-α. IL10 gene expression was observed to be higher in the group of black-and-white Holstein and Gyr cows with mastitis compared with animals free of infection in both breeds ($P < .05$), as revealed by quantitative polymerase chain reaction (PCR).

Strategies for combating mastitis using molecular genetics tools

Since the majority of these genes are expressed most during the involution stage and least during the lactating stage, these genes have gained importance. Molecular genetics for these immune-response genes may be exploited for combating the incidence of mastitis and are classified in four broad ways. The strategies include identification of the genes involved in mastitis defense and then using these candidate genes as markers for mastitis resistance in the form of marker-assisted selection (MAS). Other approaches include use of the recombinant products of these genes as therapeutics during the lactational stage, when it is least expressed. Transgenic animals produced with the gene insert will produce this antibacterial peptide in substantial amounts to prevent mastitis. Transgenic technology is of great use when the gene is normally not expressed in species suffering from the disease. For example, Lysostaphin, a prokaryotic gene of *Staphylococcus simulans,* codes for lysostaphin, which is quite effective against *Staphylococcus* mastitis. A recent approach that is very useful against mastitis is somatic gene therapy, where the insert is inoculated in, and the protein is expressed locally within, the mammary gland.

A major strategy may be improvement of the immune response or defense mechanism of the mammary gland. Thus the set of genes known to be responsible for innate immunity of the udder as well as those coding for various antibacterials, including various cytokines, may be used as candidate genes for mastitis resistance.

The most important step is identification of the gene of interest that is involved in mastitis defense. A variety of methods have been found useful. The mRNA differential display method was used to identify mastitis-associated expressed DNA sequences of the noninfected and infected udder quarters of cows. Expressed sequence tags were used to identify the sequence homology of genes involved in regulating gene expression, growth, and the differentiation factor encoding these genes as well as immune-response or inflammatory markers. Studies with quantitative RT-PCR revealed mastitis-associated expression in udder samples of animals with or without clinical mastitis. Physical mapping was done using a bovine−hamster somatic cell hybrid panel and a 5000 rad bovine whole-genome radiation panel. According to their localization in quantitative trait locus (QTL) regions based on an established marker/gene map and their disease-associated expression, four genes—AHCY, PRKDC, HNRPU, and OSTF1—were suggested as potentially involved in mastitis defense. The microarray explorer tool had been used for analyzing quantitative cDNA expression profiles for the gene of mammary gland.

*Bacterial gene.

Strategies for mastitis resistance that can be employed in a molecular approach

1. Immune-response gene used in marker-assisted selection

The main criteria for a marker in MAS are that it should be sufficiently polymorphic, abundant, and transmitted from generation to generation. Polymorphism study of the genes significantly associated with indicator traits pertaining to mastitis, such as somatic cell score (SCS), white side test, California mastitis test, etc., has found them useful against mastitis. Molecular markers are preferred over other biochemical, cytogenetic markers because they can be explored at the DNA level and can be studied irrespective of sex and age as well as from any body tissue. Molecular markers can even be used to explore variation in the coding region. Polymorphism studies with restriction fragment length polymorphism (RFLP) and single-stranded conformation polymorphism (SSCP) as type I markers, as well as close association of the desired trait of interest with a microsatellite marker, have also been found to be beneficial. SSCP in Jersey cattle for the IL8 gene had significant association with both subclinical and clinical mastitis. The beta defensin 5 encoding gene has been studied as a candidate gene for mastitis resistance. PCR-RFLP in combined defensin genotypes revealed significant association with milk SCS in black-and-white cows. Mapping of economic trait loci for SCS in Holstein cattle using microsatellite markers has been conducted followed by selective genotyping.

2. Recombinant protein as antibacterial substance

Recombinant polypeptides obtained after introgression of the insert containing the gene pertaining to the production of antibacterial substances effective against mastitis-causing organisms have been proved very effective. Intramammary infusion of recombinant protein reduces the incidence of mastitis. The main advantages of recombinant proteins are that they are easily degraded in the body system and subsequently the risk of evolving resistant pathogens is minimized. Moreover these recombinant peptides can be administered during the lactational stage, when the animal is most susceptible to the incidence of mastitis. Also the antibacterial peptide, which is not normally expressed in the species through recombinant technology, may be expressed in substantial amounts after manipulating the regulatory region. In vitro and in vivo studies indicate that rbIL2 enhances the functional capabilities of mononuclear cells within the mammary gland, and rbIL2 enhances immune response by intramammary infusion during increased risk of mastitis and when administered with antibiotics, improves mastitis resistance. Recombinant bovine neutrophil β-defensin had strong bactericidal activity against *S. aureus* and *Escherichia coli,* and it have also been suggested to be used as a therapeutic for treating mastitis.

3. Transgenic animal production against mastitis

Transgenic production of the foreign protein coding for antibacterial substances in milk has been found to be an effective and permanent means of preventing mastitis. For the first time, foreign protein was produced in mouse milk in 1987. A Jersey cow was cloned with lysostaphin gene insert by somatic cell nuclear transfer. March 2000 is regarded as the time of the first appearance of a transgenic cow resistant to mastitis, named "Annie." Genes for lysostaphin, green fluorescent protein (an antibiotic marker), and the sheep gene for β- Lactoglobulin, which served as a switch, were introgressed in the fibroblast cell nucleus. This nucleus was then introgressed in the enucleated unfertilized ovum which was then implanted as a fertilized egg and thus Annie was born. This was confirmed by genetic testing of the umbilical cord, which was transgenic. A transgenic approach to enhance disease resistance involves the production of pathogen-specific antibodies into milk. Transgenic animals producing corona virus, neutralizing antibodies into milk under the control of 5′ regulatory regions of the murine whey acidic protein gene, have been reported. In the future, this technology to enhance mastitis resistance may be helpful for the generation of the appropriate hybridoma cell line. Another transgenic approach is the overproduction of the pIgR gene in mammalian epithelial cells, conferring its resistance against mastitis. Table 19.1 lists out transgenesis for disease resilience and other production traits.

4. Somatic gene therapy against mastitis

Somatic gene therapy has been proved a valuable and effective technique for prevention and cure in cases of mastitis. The basic principle in somatic gene therapy is that appropriate cells are taken from the organism, and a healthy gene is introduced into them. Afterward, the cells are reimplanted into the organism. This kind of therapy is thus characterized by the genetic alteration of normal body cells. These cells are supposed to restart their normal function expressing the "healthy" gene.

In gene therapy, vectors derived from various viruses are tested for the transfection of cells because they may easily introduce their genome into host cells. It cannot be totally excluded, however, that under somatic gene

TABLE 19.1 Transgenesis for disease resilience and other production traits.

Gene modification and transgenic traits	Species	Transgene
Increased growth	Pig	GH and IGF1
Larger ratio of n-3 to n-6 fatty acids	Pig	Fat1
Reduction of the environmental impact through phosphorus and nitrogen release reduction	Pig	Phytase
Avian influenza resilience	Chicken	shRNA decoy
Mastitis resilience	Goat Cow	Lysozyme Lysostaphin
Porcine reproductive and respiratory syndrome virus resilience	Pig	Histone deacetylase

TABLE 19.2 Major histocompatibility complex genes/variants associated with VM or CAE pathogenesis.

Region	Variant	Typing method/marker	Species/breed(s) studied
Class I	Allele CLA Be7	Alloantisera	Goat/Saanen
	Allele OMHC1*205	Microsatellite	Sheep/Latxa
Class II	Alleles DRB1*0403 and DRB1*07012 and various amino acid positions	Cloning and direct sequencing	Sheep/Rambouillet, Polypay, and Columbia
	DRB1*0325	PCR sequence- based typing	Sheep/Latxa
	Allele DRB2*275	Microsatellite	Sheep/Latxa

therapy, genes could be introduced in the germ line. There are nearly no ethical objections against somatic gene therapy. In vivo transfection of the mammary gland for the production of high-value protein against the pathogens known to be responsible for mastitis has been found effective, and foreign protein production into milk was found over 60 days after in vivo transfection of lactating mammary gland of ewes with plasma DNA. Recombinant interferon was produced in the milk when the mammary gland was transfected with recombinant interferon gene construct. In an attempt for gene therapy, the mammary-specific vector containing human lysozyme (hLYZ) cDNA and the kanamycin-resistance gene was constructed. It was designed with an aim to express human lysozyme within the mammary gland in response to clinical mastitis.

Development of clustered regularly interspaced short palindromic repeat gene-editing technology for disease-resistant cows

Tuberculosis-resistant cows were developed for the first time using clustered regularly interspaced short palindromic repeat (CRISPR) gene-editing technology. As discussed earlier, the first transgenic cow with lysostaphin gene insert was developed as early as 2005. In the current era, gene editing tools are available as CRISPR technology. A novel version of the CRISPR system called CRISPR/Cas9 was successfully inserted with a tuberculosis-resistant gene, called NRAMP1, into the cow genome. Researchers were then able to successfully develop live cows carrying increased resistance to tuberculosis.

The first application of a single Cas9 nickase (Cas9n) to induce gene insertion at a selected locus in cattle was reported. The main binding sites of a catalytically inactive Cas9 (dCas9) protein in bovine fetal fibroblast cells with chromatin immunoprecipitation sequencing (ChIP-seq) was identified. Subsequently, a single Cas9n-induced single-strand break can stimulate insertion of the NRAMP1 gene with reduced but still considerable off-target effects. Through somatic cell nuclear transfer, transgenic cattle with increased resistance to tuberculosis can finally be obtained. No off-target effects on cow genetics were observed, which implies that CRISPR technology may be better suited to producing transgenic livestock with purposefully manipulated genetics. Employing this technology may be better suited to producing transgenic livestock with purposefully manipulated genetics.

A total of 11 calves with new genes inserted using CRISPR were assessed for resistance to tuberculosis and any off-target genetic effects. Genetic analysis of the calves revealed that NRAMP1 had successfully integrated into the genetic code at the targeted region in all the calves. No calves that had the gene inserted using the new CRISPR/Cas9n technology had any detectable off-target effects, whereas all calves with the gene inserted with previously used techniques for CRISPR/Cas9 did.

When the calves were exposed to *M. bovis*, the bacterium that causes bovine tuberculosis, the researchers found that transgenic animals showed an increased resistance to the bacteria measured by standard markers of infection in a blood sample. They also found that white blood cells taken from the calves were much more resistant to *M. bovis* exposure in laboratory tests.

The current study was reported to be the first demonstrating that the CRISPR/Cas9n system can be used to create transgenic livestock with no detectable off-target effects. This work has led to the discovery of a useful position in the bovine genome that can be targeted with this gene editing technology to successfully insert new genes that benefit agricultural livestock.

Development of trypanosomiasis resistant cattle at the International Livestock Research Institute- a case study

Africa has a prevalence for trypanosomiasis, transmitted by the tsetse fly. Trypanosomiasis is popularly known as "nagana" in animals and "sleeping sickness" in human beings. Previously, attempts at vaccination against trypanosomiasis had proved to be highly unsuccessful. So development of trypanosomiasis-resistant cattle is the best option. Plans are under way at the International Livestock Research Institute (ILRI) to develop a cow that is resistant to trypanosomiasis. Trypanosomiasis-resistant genes (two genes) were identified from baboons that are naturally resistant to the disease. A cloned transgenic Boran calf named "Tumaini" was developed and claimed to be resistant to trypanosomiasis. It is to be noted that the N'Dama breed of cattle native to Africa is naturally resistant to this disease.

The research that merged a range of high-tech tools and field observations has sought to find biological answers to protection from a single-celled trypanosome parasite that causes both African sleeping sickness in people and the wasting nagana disease in cattle.

According to disease-mapping scientists and economists at ILRI, the tsetse fly belt across Africa stretches from Senegal on the west coast to Tanzania on the east coast, and from Chad in the north to Zimbabwe in the south. Annually, the disease renders millions of cattle too weak to plow land or haul loads, and too sickly to give milk or to breed before the disease finally kills off most of those infected.

The clone was observed to be healthy and is being raised at Kenya's ILRI research facility. New research published by an international research team in *Proceedings of the National Academy of Sciences* and using a new and intensive combination of approaches has found two genes that may prove of vital importance to the lives and livelihoods of millions of farmers in tsetse fly-plagued Africa. ILRI estimates that the annual economic impact of "nagana" (officially known as African animal trypanosomiasis) stands at USD 4–5 billion.

It is estimated that, courtesy of increased surveillance and control in the 13 years from 1998 to 2009, sleeping sickness in people dropped from 300,000 to 30,000. During that period, it killed more than half of those it infected.

International research on the hereditary norms of disease resistance research brought together scientists from Africa, Europe, and Asia. It was led by scientists from the Nairobi-based ILRI and the universities of Liverpool, Manchester, and Edinburgh and drew the input of researchers from other institutions in Britain, Ireland, and South Korea. This may be the first example of scientists bringing together different ways of getting to the bottom of the genetics of a very complex trait.

The key informant of the research was a humpless West African breed called the N'Dama. Most African cattle, especially the Boran breed, are susceptible to disease-causing trypanosome parasites. N'Dama, on the other hand, is not seriously affected by the disease, as it was domesticated in Africa some 8000 or more years ago and as such has had time to evolve resistance to the parasites. Even though the N'Dama is a valued asset in the continent's endemic regions, it is characteristically small in size and produces less milk. However, its disease-resistant attributes are what many farmers would want transmitted to more productive breeds. The N'Dama disease-resistant gene has been the "Holy Grail" for international livestock geneticists for more than 20 years.

Genetic approaches to distinguish differences between the West African N'Dama and Boran cattle, which come from Kenya in East Africa, were used at intervals to look for differences in those sequences between the two breeds. The Edinburgh team conducted gene expression analyses investigating differences in genetic activity of the two cattle breeds after sets of animals of both breeds were experimentally infected with the parasites. Finally, they looked at the genetics of cattle populations from all over Africa.

Another case study

One of the emerging diseases affecting cattle health is bovine respiratory disease (BRD). It has been recorded as one of the important diseases of high mortality. This disease was recorded as a major cause of death in preweaned calves in the United States and is responsible for half of all feedlot deaths. In an analysis of 20 years' data of the US Meat Animal Research Center involving 43,739 calves, 10.5% were diagnosed with BRD.

A West Texas A&M University veterinary epidemiologist indicates that BRD, also referred to as shipping fever, or undifferentiated fever in Canada, is the leading cause of death, loss, and chronic illness in US feedlots. In fact, it affects more animals than all other diseases combined and costs the industry more than $600 million annually. Heritability for disease occurrence over all diseases was reported to be 0.28 in New Zealand, easily high enough for selection. Morris predicted these heritability estimates as grass tetany, 0.36; milk fever, 0.39; pinkeye, 0.28; and BRD, 0.19. Statistically significant breed differences in incidence and mortality of preweaning and feedlot BRD were reported. Estimated heritability of resistance to BRD in preweaned calves was 0.14, and in feedlot calves 0.18, probably high enough for effective selection.

Resistance or adaptability?

It is evident that when we select healthy animals, presumed free from disease, they are selected for innate immunity and stress tolerance. Although we assume that we are selecting for disease tolerance, animals are automatically selected for more tolerance to stress. Selection based on mere phenotype includes selection for innate immunity as well as stress tolerance. A particular disease result from the interaction of stressors, pathogens, and animal susceptibility, but stressors have the biggest effect.

What are the challenges?

Before a trait can be selected, a measurable and observable phenotype is necessary. In addition, data on the phenotypes must be monitored and collected with reasonable cost and effort. If sufficient phenotypic variation exists in the population and heritability is moderately high, genetic progress can be made by selecting animals that exhibit the desirable phenotype.

Many traditional expected progeny differences were developed and are updated in large part by using data submitted by purebred breeders or collected by breed associations. But unlike weighing calves, can producers correctly identify disease phenotypes?

Hence it can be concluded that BRD disease resistance depends on

1. Innate immunity
2. Stress
3. Body weight

Disease resistance for sheep and goats

Sheep and goats form the main basis for meat production in India. Small ruminants such as sheep and goats form the main assets for marginal and landless laborers. Farmers generally rear goats and sheep together in a flock. In a separate study conducted on disease incidence in the Birbhum district of West Bengal, it was observed that sheep are mostly resistant to parasitic infestation and other diseases, especially peste des petits ruminants (PPR). So there is a scope for exploring the genetics of disease resistance in sheep and the characterization of disease resistance genes in different breeds of sheep.

The common diseases affecting the sheep population in West Bengal are parasitic infestations, particularly *Haemonchus* spp. and immature amphistomes. Sheep are also affected by common respiratory diseases.

CSIRO, Australia has exploited the development of a disease-resistant Merino flock. Diseases for sheep and goats include gastrointestinal nematode infections, diseases due to mycotoxins, bacterial diseases including foot rot and mastitis, ectoparasites such as flies and lice, and scrapie, the small ruminant transmissible spongiform encephalopathy.

It has been documented that individual genetic variations exist for animals in terms of disease resistance. Genetic variations have also been detected among sheep—goat populations in terms of infectious diseases. These variations were observed to be heritable. These heritable differences may be employed for breeding animal with better resistance to diseases. Studies have been conducted for some diseases such as nematode infestation, resistance to fly-strike and myiasis, and mycotoxin poisoning. Experimental flocks were utilized for studying the feasibility of breeding for disease resistance. For some economically important diseases such as nematode parasitic infestation, mastitis, foot rot, scrapie, and mycotoxin poisoning, selection against these diseases has already been introduced in breeding programs. Most success has been obtained in sheep rather than goats.

Research is ongoing for the identification of genetic markers associated with resistance to diseases, including infectious diseases. The approach is to select animals free from the respective disease through MAS or genomic selection. Functional genomics is of importance for studying disease resistance, which includes gene mapping and gene expression studies. A recent approach for breeders to select sheep and goats for better disease resistance is integrated studies including quantitative and functional genomics and large-scale data collection (within and between breeds) combined with epidemiological prediction. Although most disease traits are multitrait, recently discovered single genes influencing disease resistance include the prion protein (PrP) gene related to scrapie susceptibility in sheep.

Genetic resistance to diseases in goats—genomic insights

Gastrointestinal nematode infection

Gastrointestinal nematode infection is a major problem in the small ruminant industry worldwide. Susceptibility to nematode infection seems to be related to genetic factors, and resistance may vary among breeds. Comparative studies have shown that goats are more susceptible to gastrointestinal nematodes than sheep. The recommended drug dosage to goats is the same as for sheep, but due to differences in the pharmacokinetics of drugs between sheep and goats, anthelmintics are less efficacious in goats and may lead to rapid selection of anthelmintic-resistant worms.

MHC is a candidate gene considered important for the immune system and disease-resistant traits. MHC is a cell surface molecule involved in antigen presentation by glycoprotein receptors of immune cells (B lymphocytes, dendritic cells, and macrophages).

MHC is divided into three subgroups: class I, class II, and class III. The length of MHC I in goats is 1077 bp, encoding a mature protein with 337 amino acids. MHC class II molecules can be separated into DQ and disease-resistance (DR) subtypes and probably play a major role in the development of MHC-restricted immune responses. In ruminants, DRB is the most polymorphic locus of the MHC gene complex having significant association with the resistance to nematodes.

The DRB1*1101 exon 2 encodes the β1 domain, which makes up part of the PBR (peptide-binding region) of DR molecules. The residues found in this region have close contact with the peptides present in the PBR or T cell receptor region. Therefore, these regions are likely to have roles in disease resistance or susceptibility. Gene expression of DRB1*1101 was elevated in meat breeds of goats after being exposed to *Haemonchus contortus*. Gene expression was highest in Boer goats, lower in Myotonic goats, and lowest in Spanish goats. Gender differences in DRB1*1101 expression were observed, where males showed higher expression than females. Age differences influenced DRB1*1101 gene expression. The DRB1*1101 allelic expression was higher in younger (<4 years old) than older (>4 years old). Overall, regardless of breed, DRB1*1101 expression was higher in naturally susceptible than in resistant groups of pasture-infected goats when compared. The packed cell volume (PCV) was somewhat negatively correlated (47%) with DRB1*1101 expression, indicating that DRB1*1101 was more correlated with susceptibility to *H. contortus* than with resistance. Three genotypes in the *Taq*I locus and two genotypes in the *Bsa*I locus were identified in the MHC Class II DRB gene in Rohilkhand goats. The loci were observed to follow Hardy—Weinberg equilibrium. It was predicted to follow a balancing selection at the MHC loci as evidenced by a high degree of variability.

Studies in Kenya with *H. contortus* and in France with gastrointestinal strongyles have shown the existence of genetic variation in the susceptibility of goats to nematode infections. However, in a study in Fiji, very little genetic variation in mixed *H. contortus* and *Trichostrongylus colubriformis* infections in goats were found. Thai Native goats were found to be more resistant to *H. contortus* for parasitological and blood parameters, as they had lower eggs per gram of feces, lower worm counts, and lower reduction in blood values compared with their Anglo—Nubian crosses.

Single-nucleotide polymorphisms (SNPs) pertaining to four candidate genes of the cytokine family (IL2, IL4, IL13, and IFNG) were studied that might be associated with resistance to gastrointestinal endoparasites in Saanen and Anglo−Nubian goats. Of the 10 SNPs, 3 were identified as significant ($P \leq .03$). They were found in intron 1 of IL2 (ENSBTA00000020883), intron 3 of IL13 (ENSBTA00000015953), and exon 3 of IFNG (ENSBTA00000012529), suggesting an association with gastrointestinal endoparasite resistance.

Coccidiosis

Coccidia infections are one of the major causes of kid mortality and cause severe economic losses to goat production by affecting the early growth phase of kids. The causal agent for coccidiosis infection is *Eimeria* species, and it is host-specific. Sixteen *Eimeria* species have been described from goats worldwide. The major coccidia species that were found in the semiarid region of India were *Eimeria christenseni*, *Eimeria jochejevi*, *Eimeria ninakohlyakimovae*, and *Eimeria arloingi*, with reported genetic variability in resistance. Breed-wise differences were observed for oocyst counts at 3 months of age between Jamunapari and Barbari goats. Breed differences have been observed between crossbred and indigenous breeds of South Africa. The fecal oocyte count at both 3 and 6 months of age was negatively phenotypically correlated with both live weight and live weight gain in Jamunapari and Barbari goats. Therefore, the fecal oocyst count between 3 and 6 months of age should be considered for genetic susceptibility analysis. However, before implementing these results in a breeding program, it is necessary to first estimate more precise genetic parameters in a larger population.

Peste des petits ruminants

PPR is an important disease of goats responsible for mortality. Etiology is PPR virus (PPRV), a morbillivirus of the family Paramyxoviridae. Breed-wise differences in susceptibility to PPR have been observed in goats. West African goats (dwarf goats) were observed to be more susceptible to PPR than were European breeds. Breed-wise variation in susceptibility was also observed in Indian breeds of goat. Variation in the susceptibility among the host may be due to the differential presence or distribution of specific viral receptors. These may be the signaling lymphocyte activation molecule that has previously been observed to be associated with PPRV and other morbilliviruses such as measles and canine distemper. TLRs are another class of molecule responsible for host innate immunity. TLR1 has an active role in the innate immune system. It is a transmembrane protein that acts through pathogen-associated molecular patterns (PAMPs). TLRs that act intracellularly include TLR3, TLR7 and TLR8 and TLR9 (CpG motifs in DNA). Cytokine profile in response to ligand has important role in innate immunity. TLR7 of goats was observed to have important role in resistance against PPR, a devastating viral disease. It is 3.4 Kb long, with a 3141-nucleotide open reading frame coding for 1046 amino acids. Synonymous mutations were detected: five SNPs in the coding region and two SNPs in the 3′ UTR.

Scrapie

Scrapie is an infectious neurodegenerative disease caused by accumulation of an aberrant isoform of normal PrP (i.e., conversion of a normal cellular protein, PrPC, into the abnormal isoform PrPSc) due to infection. It was observed that alterations in the host gene that encodes PrP are responsible for the occurrence of scrapie. The details were described in Chapter 2.

Polymorphism has been detected in PrP genes of goats at 142, 143, 154, 222, and 240 bp associated with scrapie susceptibility. The polymorphic site at 142 bp was responsible for an altered disease incubation period. In goats, coding mutations of the gene encoding the PrP (I/M_{142}, N/D_{146}, S/D_{146}, R/Q_{211}, and Q/K_{222}) were observed to be associated with a lower risk of developing classical scrapie. Haplotype 1 was the only haplotype observed in every breed examined. Haplotype 2 was common in all breeds except Toggenburg and Myotonic. Haplotypes 3, 4, 5, 8, 9, and 10 were observed only within dairy breeds. Another three PRNP variants have been associated with incomplete scrapie resistance and were observed in US goats. The M142 variant has been associated with extended scrapie incubation time and had previously been found in Saanen and mixed-breed dairy goats. Further study is necessary to demonstrate full genetic scrapie resistance before genetic approaches to goat scrapie can be helpful in current efforts at scrapie eradication.

Genomic insight for disease resistance in sheep

A detailed discussion of genomic studies in terms of immune-response genes was covered in Chapter 7. Some other factors are described here.

Small ruminant lentiviruses (SRLVs) are important for both sheep and goats. SRLVs belong to the retrovirus family. They are closely related to visna–maedi virus (VMV) and the caprine arthritis-encephalitis virus (CAEV), which infect sheep and goats. Both infect cells of the monocyte/macrophage lineage and cause lifelong infections.

In this disease, proviral DNA transcription and gene expression are suppressed until infected monocytes mature into macrophages. In the target organ, infected macrophages start replication of the virus including an inflammation cascade. In spite of the immune response present, the virus is not expelled, and the host will act as a lifelong carrier. Host genetic factors, infecting viral strain, and management influence the occurrence, length, and spectrum of the affected organ. In addition to classical breeding experiments, studies with advanced genomic techniques are in vogue. Breed-wise disease susceptibility has been observed in Karakul sheep thought infected with VMV. Sheep with coarse wool were observed to be more susceptible than those with fine wool. For example, Border Leicester sheep were observed to be more susceptible than Columbia sheep to developing SRLV-specific lesions.

Host genetic factors involved in small ruminant lentivirus-induced pathogenesis

Factors influencing SRLV pathogenesis include different strains of pathogen, differential disease progression, host species and breeds, and affected organ spectrums. It seems that the relationship between small ruminants and SRLVs is complex and that pathogenesis is likely induced by a number of genes with small or moderate effects. The cellular receptors for SRLVs have not been conclusively identified and therefore that information cannot yet be exploited. Most likely it is a common cell membrane molecule, as SRLVs can enter other cells apart from target cells and thus the receptor does not dictate cell tropism. It was observed that classical VMV and CAEV strains appear to use different receptors. The mannose receptor (MR) is a putative receptor for SRLVs. Researchers have characterized the ovine MR nucleotide and protein sequence, and its role in VMV infection is currently being studied. Both innate and humoral immunity are involved in providing immunity against SRLV infection. Host genetic factors that have been involved in SRLV infection and disease are discussed in the following sections.

Major histocompatibility complex

The role of MHC has been depicted in SRLV infection and SRLV-induced disease. The MHC locus was observed to be located on chromosome 20 in sheep and chromosome 23 in goats. MHC Class I and II genes have the role of binding and presenting antigenic peptides to T cells, in turn initiating immune response. Its polymorphism and association with infectious disease have been intensely studied in a myriad of diseases with viral etiology, although such studies are scarce in small ruminants.

In the ovine species, VMV infection increases the expression level of MHC Class II in the lungs, CNS, and synovium. It was observed that Class I and II polymorphic gene variants were associated with SRLV provirus levels and disease progression. Many loci in the MHC have a highly polymorphic nature, among which the DR beta 1 (DRB1) gene is a clear example (over 100 alleles have been identified to date in sheep). Table 19.2 discusses major histocompatibility complex genes/variants associated with VM or CAE pathogenesis in sheep and goat.

Antibody and T cell response

Humoral immunity due to SRLV has been well studied with antibody and T cell response through serologic diagnostics.

Neutralizing antibodies were observed to be slow in induction or have low affinity and relatively low titers. However, it was found that propagation and spread cannot be fully stopped by antibodies because a small number of free viruses were observed to spread from cell to cell. Some antibodies were observed to have better response, while others were found to be skewed in SRLV-infected sheep and goats. Only immunoglobulin G1 (IgG1) subtype responses are detected in sheep, while in goats IgG1 dominates humoral response.

Both T cell proliferative and cytotoxic responses were found to be induced after SRLV infection. The CD4+/CD8+ cell ratio was reduced in the bronchoalveolar, synovial, and cerebrospinal fluids of diseased animals because of higher levels of CD8+ cells, which may be due to the severity of lung lesions. Cytotoxic T lymphocytes appear to be important effector cells, for they may inhibit viral replication but also contribute to lesions through cytokine production or cytotoxicity. In ruminants, IgG1 induces a type-2 T helper cell (Th2) response, and IgG2 induces Th1 response.

It is interesting to note that goats with a Th2-biased CD4+ T cell response developed arthritis, but in goats with a mixed IgG1 and IgG2 response, no clinical signs were observed. These facts suggest that anti-SRLV IgG2 antibodies protect against disease induction. In addition, CD80 levels, which favor Th1 cell differentiation, were low in clinically affected sheep compared with those of asymptomatic and seronegative controls.

Cytokines and receptors

Cytokines are important for signaling and mediating immune response and have a major role in SRLV-induced pathogenesis. Some cytokines modulate ongoing immune activation in target organ lesions, while others favor the appearance of lesions. Cytokines induce the recruitment and differentiation of monocytes to macrophages as well, favoring additional infection by attracting target cells and thus creating a vicious circle.

Although most research has focused on expression, a few works have explored the influence of polymorphisms. Cytokine polymorphisms are known to affect cytokine gene transcription, or they may affect cytokine function and thus influence the outcome of infection and disease. Chemokine receptor 5 (CCR5) polymorphisms could affect SRLV pathogenesis by participating in leukocyte recruitment against different pathogens, including ovine lentiviruses. A deletion mutation causes an almost fourfold reduction in transcription in animals carrying the deletion allele as well as showing association with decreased provirus burden. Considering that monocytes/macrophages are the main target cells of VMV, it has been suggested that reduced chemotactic ability of CCR5 could result in reduced influx of such cells to the site of infection, which could also slow the rate of cellular infection. Alteration of the cytokine profile has been suggested in both sheep and goats, and it is suspected that SRLVs induce a switch to a Th2-type cytokine response. Evidence backing this is the upregulation of type 2 cytokines such as antiinflammatory cytokines IL4 and IL10.

Genes such as TLRs and their polymorphisms were observed to have a significant role and be good candidate genes in various diseases affecting domestic livestock. TLRs are a family of transmembrane signaling molecules that trigger both the innate and adaptive immune-response mechanisms in response to PAMPs, including viral components (Table 19.3).

Individual susceptibility to diseases was observed in the Tsigai breed, which may be defined by the presence of SNPs in TLR7 and TLR8 sensing double-stranded viral RNA. These two genes have recently been found to be upregulated in diseased sheep.

In response to retroviruses and other transposable elements that invade the host's genome, hosts develop intracellular defenses known as restriction factors. These proteins block the viral cycle using different strategies, such as directly attacking viral structures or editing viral genetic material during reverse transcription.

Ovine tripartite motif protein 5 alpha (TRIM5α) seems to be able to restrict VMV DNA synthesis. This intracellular factor restricts retroviral replication by interacting with the capsid and preventing uncoating. A recent study has suggested an association between TRIM5α polymorphism that inactivates antiviral activity and accelerates AIDS disease progression. Apolipoprotein B mRNA-editing enzyme (APOBEC3) proteins, which act against a wide variety of retroviruses including HIV, have been studied in sheep and could be good candidates for SRLVs. A human APOBEC3G variant has been associated with rapid HIV-1 disease progression. Recently, allele and haplotype variants of the transmembrane protein gene 154 (TMEM154) locus have been associated with SRLV infection and has been proposed as a locus that could be used for genetic marker-based selection. Sheep with the ancestral variant haplotypes of this gene have an increased risk of becoming infected compared with individuals with mutant forms. However, the function of TMEM14 has not yet been established.

In our lab, we have studied the role of various cytokines with roles in providing parasitic immunity. Studies included SNP detection and polymorphism analysis of the genes (IL10, IL1B, NFκβ, IL12, IL6, CD14, TLR4, myeloid differentiation primary response gene 88 (MyD88), MD2, and LPB). Differential mRNA expression analysis for these genes was observed with respect to infected (with *H. contortus*) and noninfected sheep. The details were discussed in Chapters 7 and 13.

TABLE 19.3   Cytokine and cytokine-associated genes implicated in SRLV pathogenesis in sheep and goats.

Gene symbol	Gene	Species	Methods	Analyzed material	Parameter analyzed
ILlβ	*Interleukin-1 beta*	Sheep	Semiquantitative RT-PCR	Lung	Clinical disease
IL2/IL2R	*Interleukin-2/Interleukin-2 receptor*	Sheep	Semiquantitative RT-PCR, qPCR	Lung, PBMCs, lymph node leukocytes	Infection, clinical disease
		Goat	In situ hybridization	Joints	Infection, clinical disease
IL4	*Interleukin-4*	Sheep	Semiquantitative RT-PCR	Lung	Clinical disease
		Goat	Semiquantitative RT-PCR	PBMCs	Clinical disease
IL6	*Interleukin-6*	Sheep	RT-PCR	Alveolar macrophages	Infection, clinical disease
IL8	*Interleukin-8*	Sheep	RT-qPCR, in situ hybridization, northern blot, semiquantitative RT-PCR	BALF, alveolar macrophages	Infection, clinical disease
		Goat	In situ hybridization	Macrophages	Infection
IL10	*Interleukin-10*	Sheep	Semiquantitative RT-PCR	Lung, alveolar macrophages	Infection, clinical disease
IFNy	*Interferon-gamma*	Sheep	Semiquantitative RT-PCR	Lung	Clinical disease
		Goat	Semiquantitative RT-PCR, in situ hybridization	PBMCs, joints	Clinical disease
TNFα	*Tumor Necrosis factor-alpha*	Sheep	qPCR	Lung, udder	Clinical disease
TGF-β1	*Tumor growth factor-beta 1*	Sheep	Semiquantitative RT-PCR	Alveolar macrophages	Infection, clinical disease
		Goat	In situ hybridization	Macrophages	Infection
MCP-1	*Monocyte chemoattractant protein 1*	Goat	In situ hybridization	Macrophages, joints	Infection, clinical disease
GM-CSF	*Granulocyte macrophage stimulating factor*	Sheep	Semiquantitative RT-PCR	Lung, alveolar macrophages	Infection, clinical disease
		Goat	In situ hybridization	Macrophages	Infection
CCR5	*Chemokine (C-C motif) Receptor 5*	Sheep	Cloning & sequencing, qPCR	PBMCs, lung	Infection, clinical disease

RT-PCR, real-time polymerase chain reaction; *qPCR*, quantitative polymerase chain reaction; *PMBC*, peripheral blood mononuclear cell

A case study by the International Atomic Energy Agency on the topic "Genetic Variation on the Control of Resistance to Infectious Diseases in Small Ruminants for Improving Animal Productivity"

DR was observed to be a heritable trait, hence it is possible to select DR traits for animals with enhanced resistance for diseases. Successful breeding programs have been advocated for selection of healthy sheep with the help of genetic markers for scrapie and nematode parasitism. Genetic disease resistance is usually more important in developing countries, as indigenous breeds possess enhanced resistance to local diseases compared with exotic ones reared in the same environment. The characterization and mapping of genes controlling "quantitative trait loci," SNP markers, and the subsequent use of this information in selection and breeding programs is important to increases in small ruminant economic traits.

"Gene-based Technologies in Livestock Breeding: Phase 1 − Characterization of Small Ruminant Genetic Resources in Asia" has provided relevant information on which this CRP project proposal is building. Through this CRP, technical capacity within the eight Asian countries was notoriously improved, and many scientists from the region were trained on the use of radioisotopic microsatellite methods and related technologies for genotype characterization of ruminants, and equipment for the laboratories was procured through the project. Genotypic and phenotypic information were collected and partially analyzed from 40 sheep breeds and 60 goat breeds. The new CRP will use much of the collected data.

The analysis of mutations (SNPs) is in essence the discovery and exploitation of the natural variation existing in the biological machinery with the aim of increasing the frequency of favorable alleles to the benefit of livestock breeding strategies.

The two main research trials designed for the first phase of the CRP were the artificial challenge and the field challenge. For the first trial, a minimum of 20 animals per breed will be experimentally infected with a dose of 5000 L3 of *H. contortus*, and phenotypic data and a DNA sample will be obtained while animals are kept in pens or drylots with minimal access to parasite-contaminated feeds; whereas in the field challenge, over a period of 2 years, a minimum of 500 animals of the susceptible breed, or 200 animals per breed if two breeds are selected, will be naturally infected while grazing, and phenotypic data and DNA samples will be collected. In both cases, animals will be ear tagged and should be 4–6 months of age at the time of sampling. Body weight, fecal egg counts (FECs), PCV, and FAMACHA scores will be monitored among other phenotypic variables. Agreement holders presented up-to-date information related to molecular markers, genome-wide analysis, genomic selection and parasite resistance in breeding programs. In addition, a full day was devoted for lectures by IAEA and FAO staff, agreement holders, and invited observers on procedures for data collection, use of FAMACHA, DNA extraction and quality control, sample identification and storage, experimental design, and collaborative work. These presentations generated fruitful discussions that clarified steps and activities to be carried out during the first phase of the project.

The first step is the identification of (at least) two breeds of sheep or goats, where one is suspected of being "resistant" to gastrointestinal parasites. Animals should be 4–6 months of age at the time of data collection and sampling. The second step is the quantification of relative resistance to gastrointestinal parasites of sheep and goat breeds using an artificial challenge protocol and then a natural field challenge.

The "Artificial Challenge" trial will consist of a minimum of 20 animals per breed of equal sex distribution that are brought to a common location and dewormed. Then, 4–6 weeks later a blood sample for DNA extraction will be collected and animals challenged with 5000 infective L3 larvae. Body weight, FECs, PCV, and FAMACHA scores are taken at 28, 35, and 42 days after artificial infection. This trial can be omitted if samples and data exist from previous studies.

The "Field" trial will require relatively large numbers (several hundreds) of animals to determine genetic associations between DNA markers and parasite resistance, but these data can be accumulated across several farms and years. The trial can be done using only one breed (500 animals or more) or using two or more breeds (200 animals or more per breed in a common grazing environment). Animals will be dewormed and 28 days later body weight, FEC, PCV, and FAMACHA scores will be taken twice, 1 week apart. A blood sample for DNA extraction will be collected at the first sampling time. The size of individual flocks should be large enough to monitor and sample no less than 20 lambs/kids of each breed per year (the ideal situation is only one large flock).

DNA samples will be sent to the IAEA Seibersdorf laboratories (preferably) or at least to a commercial service provider for SNP genotyping. IAEA will also evaluate and advise on the quality of DNA purification done by each participant.

Activities related to the second phase (2013–15), such as genotyping strategies, validation of SNP markers, and data analysis, were discussed at the second RCM (2013). Also, training on genomic analysis and bioinformatics was provided during the meeting. SNP genotyping was outsourced after the second RCM. A workshop of genomic data analysis was organized after receiving the genotyping results to perform SNP x phenotype association studies. Some of the activities in the final 2 years include validation, gene sequencing, use of a low-density SNP panel, and other tools.

Disease resistance for pigs

Raising pigs that are healthy and disease free is a goal of every pork producer. Biosecure facilities are essential, yet enteric and respiratory diseases persist. Porcine reproductive and respiratory syndrome (PRRS), the associated porcine respiratory disease complex, and enteric diseases continue to cause major economic losses worldwide. It is essential to explore the host genetics to obtain a disease-resistant stock for pig production.

Mapping pig genes

Pig genetic maps have been developed internationally (see www.genome.iastate.edu/maps/pigbase.html). In current days, emphasis have been undertaken to explore the genetic variation in genes (SNP, insertion, or deletion) and its association with economic traits. Estrogen receptor alleles and porcine stress syndrome are examples. Yet for disease work, very limited progress has been undertaken.

Mapping bacterial diarrhea resistance

Significant progress was undertaken on the development of disease-resistant pigs against bacteria-induced diarrhea (*Escherichia coli*). Their resistance was due to a total lack of expression of the intestinal *E. coli* K88 receptor. Pigs without this receptor do not bind the bacteria as it passes through their intestine; thus, they are completely resistant to *E. coli* K88 infection.

In India, apart from a very few commercial farms, pigs are reared mostly by tribal populations, and this has a major effect on socioeconomic status and improving their livelihoods.

Neonatal diarrhea in pigs is an important problem in pig production throughout the world. The main etiological agents in neonatal diarrhea are *E. coli* strains possessing the *K88 antigen*. Single genes influencing disease resistance, including the fimbriae F4 (K88) gene in swine for reducing *E. coli* intestinal infection, have been identified. The presence of the receptor that promotes the adherence of K88*ac* pili is believed to be dominantly inherited, whereas the inheritance of the receptors for K88*ab* and K88*ad* is less clear. The use of receptor-free boars might be beneficial in herds with diarrhea problems. Lack of adherence to intestinal tissues resulted in the identification of the K88 adhesin or the genetic locus, K88. The K88 gene of pigs was localized on swine chromosome 13 (SSC13). There is a paucity of information on publicly available, quick molecular test or detection kit to identify K88-resistant pigs.

The presence or absence of *E. coli* F18 (an intestinal receptor) was responsible for providing resistance to bacterial infection. Molecular research showed that this receptor was associated with alleles of the FUT1 gene on chromosome 6.

A molecular test for FUT1 alleles was developed by Polish researchers (1999) and demonstrated that allele inheritance was associated with resistance to postweaning diarrhea due to *E. coli* F18 infections.

Work at the Immunology and Disease Resistance Lab showed preliminary data for the genetic basis of resistance to foodborne parasite infections, *Trichinella spiralis* and *Toxoplasma gondii*.

German researchers are attempting to map genes associated with pseudorabies virus resistance. Differences in susceptibility/resistance against the parasite *Sarcocystis miescheriana* were studied in the European Pietrain and Chinese Meishan pig breeds.

Case study: salmonella resistance

Recent studies in England on mapping resistance and susceptibility genes for salmonellosis illustrate the difficulty of most infectious disease mapping studies. Studies were in progress for identifying salmonella-resistant and salmonella-susceptible breeding stock to develop a reference family. All progeny were challenged with a defined dose of salmonella bacteria. Data (phenotypes) were collected on pig immunity to infection, including serum and blood cell activity and tissue bacterial burden. Additionally, genomic mapping information (genotypes) on each pig still had to be generated. Roles for phenotypic markers in resistance to salmonellosis (including blood neutrophil function and cell proliferation) were depicted. Indeed, when some pigs bred to be resistant turned out to be relatively susceptible, the potential complexity of genetic inheritance of resistance was revealed. Salmonellosis, like most infectious diseases, will likely require favorable alleles at several genes to generate substantial disease resistance.

However, the genetic test for identifying these resistant pigs was very difficult. They could be identified either by their ability to live through an infectious episode or by a bacterial binding test with pieces of their intestines after death. Lack of adherence to intestinal tissues resulted in the identification of the K88 adhesin or the genetic locus, K88.

Genetic mapping of disease immunity

Direct mapping approaches have been used to define genetic markers associated with immunity and disease resistance. Iowa State University researchers proved that different vaccine responses could be attributed to pig genetics.

Scientists genetically engineer pigs immune to costly disease. **Gene-editing technology** could be propelled into commercial farms within 5 years.

Scientists have genetically engineered pigs to be immune to one of the world's most costly animal diseases, in an advance that could propel gene-editing technology into commercial farms within 5 years.

A case study

Gene editing technology against porcine reproductive and respiratory syndrome

The trial, led by the University of Edinburgh's Roslin Institute, showed that the pigs were completely immune to PRRS, a disease that is endemic across the globe. PRRS prevalence imparts an economic loss to the European pig industry of nearly £1.5bn in pig deaths and decreased productivity each year. Pigs infected with PRRS are safe to eat, but the virus causes breathing problems for the animals and piglet mortality as well as possibly causing pregnant sows to lose their litters. There is no effective cure or vaccine, and despite extensive biosecurity measures, about 30% of pigs in England are thought to be infected at any given time.

After deleting a small section of DNA that leaves pigs vulnerable to the disease, the animals showed no symptoms or trace of infection when intentionally exposed to the virus and when housed for an extended period with infected siblings, known as *gene editing*.

Genetically modified animals are banned from the food chain in the UK and throughout Europe but it is not clear whether these regulations would apply to gene-edited animals, because the technology and resultant genetic changes are different. It is hopeful that gene editing will overcome the imitations of genetically modified organisms. Gene editing differs from older genetic modification techniques, which often involve transferring genes from one species to another. By contrast, gene editing uses precise molecular tools to remove small stretches of DNA or alter single letters in the genetic code—effectively speeding up processes that could occur naturally over many generations.

Gene editing techniques in PRRS-resistant pigs were developed by deleting 450 nucleotides, causing a receptor, called CD163, that is present on the outside of pig cells to be made lacking one tiny, precise segment that the virus binds to. This means the virus bounces off the cell rather than entering it and multiplying. Porcine reproductive and respiratory syndrome virus (PRRSV) has a narrow host cell tropism limited to cells of the monocyte/macrophage lineage. CD163 protein is expressed at high levels on the surface of specific macrophage types, and a soluble form is circulating in blood. CD163 has been described as a fusion receptor for PRRSV, with the scavenger receptor cysteine-rich domain 5 (SRCR5) region having been shown to be the interaction site for the virus. As reported previously, genetically edited pigs were developed in which exon 7 of the CD163 gene has been deleted using CRISPR/Cas9 editing in pig zygotes. These pigs express CD163 protein lacking SRCR5 (ΔSRCR5 CD163) and show no adverse effects when maintained under standard husbandry conditions. Not only was ΔSRCR5 CD163 detected on the surface of macrophage subsets, but the secreted, soluble protein can also be detected in the serum of the edited pigs, as shown here by a porcine soluble CD163-specific enzyme-linked immunosorbent assay (ELISA). Previous results showed that primary macrophage cells from ΔSRCR5 CD163 animals are resistant to PRRSV-1 subtypes 1, 2, and 3 as well as PRRSV-2 infection in vitro. Here, ΔSRCR5 pigs were challenged with a highly virulent PRRSV-1 subtype 2 strain. In contrast to the wild-type control group, ΔSRCR5 pigs showed no signs of infection and no viremia or antibody response indicative of a productive infection. Histopathological analysis of lung and lymph node tissue showed no presence of virus-replicating cells in either tissue. This shows that ΔSRCR5 pigs are fully resistant to infection by the virus.

PRRSV is the etiological agent of PRRS, causing late-term abortions, stillbirths, and respiratory disease in pigs and incurring major economic losses to the worldwide pig industry. The virus is highly mutagenic and can be divided into two species, PRRSV-1 and PRRSV-2, each containing several subtypes. Current control strategies mainly involve biosecurity measures, depopulation, and vaccination. Vaccines are at best only partially protective against infection with heterologous subtypes and sublineages, and modified live vaccines have frequently been reported to revert to virulence. Here, we demonstrate that a genetic-control approach results in complete resistance to PRRSV infection in vivo. CD163 is edited to remove the viral interaction domain while maintaining protein expression and biological function, averting any potential adverse effect associated with protein knockout. This research demonstrates a genetic-control approach with potential benefits in animal welfare as well as to the pork industry.

Another case study

Gene editing technology against the transmissible gastroenteritis virus

Transmissible gastroenteritis is a disease caused by coronaviruses and is a highly contagious and widespread virus. These viruses are known for their distinctive microscopic halos and for causing a variety of deadly intestinal diseases in livestock with almost 100% mortality in young pigs.

An enzyme called amino peptidase N (ANPEP) was detected, which acts as a potential receptor for the virus. Hence if gene editing for the gene responsible for making the ANPEP enzyme is performed, it results in a litter of pigs with a "null" gene that does not produce the enzyme. In turn, when that pig with gene edition is exposed to the transmissible gastroenteritis virus (TGEV), the pigs do not become infected. This proves that the presence of the ANPEP enzyme is necessary for infection, and gene editing can create pigs that are resistant. This study was conducted at Kansas State University.

In comparison with the scores of gene mutations that occur naturally during the reproductive process, researchers only altered the expression of a single gene. Those pigs lacking the enzyme were healthy and experienced no changes in development.

The study follows a similar success achieved in 2015, when researchers made pigs resistant to the deadly and costly PRRSV using gene editing.

The University of Missouri has partnered with Genus plc to commercialize this method of producing virus-resistant pigs, which will improve animal health and well-being and greatly reduce losses in livestock production worldwide due to viral infections, making global pig farming more sustainable. Genus plc is currently seeking FDA approval for the use of gene editing technology for eradicating the PRRSV.

The alphacoronaviruses, TGEV and porcine epidemic diarrhea virus (PEDV), are sources of high morbidity and mortality in neonatal pigs, a consequence of dehydration caused by the infection and necrosis of enterocytes. The biological relevance of ANPEP as a putative receptor for TGEV and PEDV in pigs was evaluated by using CRISPR/Cas9 to edit exon 2 of ANPEP resulting in a premature stop codon. Knockout pigs possessing the null ANPEP phenotype and age-matched wild-type pigs were challenged with either PEDV or TGEV. Fecal swabs were collected daily from each animal beginning 1 day prior to challenge with PEDV until the termination of the study. The presence of virus nucleic acid was determined by PCR. ANPEP-null pigs did not support infection with TGEV but retained susceptibility to infection with PEDV. Immunohistochemistry confirmed the presence of PEDV reactivity and absence of TGEV reactivity in enterocytes lining the ileum in ANPEP-null pigs. The different receptor requirements for TGEV and PEDV have important implications in the development of new genetic tools for the control of enteric disease in pigs.

Candidate gene identification by genetics and omics approaches in pigs

Porcine genome research is progressing with the development of genomic-based tools for the selection of livestock for disease resistance/susceptibility and improved health traits. Sufficient variability has been observed for disease traits, and disease traits were found to be heritable. Apart from classical and quantitative genetics, researchers are studying disease resistance at the genomic level through molecular approaches. These include MAS, QTL mapping, and genome-wide association studies (GWASs) to understand genetic control of host resistance to various causal agents. Most of the focus was imparted on gram-negative bacilli infections such as *E. coli*, *S. enterica*, *A. pleuropneumoniae*, and *H. parasuis*, whereas few have been done on other kind of bacilli. *S. choleraesuis* and *S.* Typhimurium are the most commonly isolated serovars that affect performance in pigs. Various pathways that are involved in both innate and adaptive IRs and associated with infections are the NF-KB pathway, antigen-presentation, ERK1/2 activation, and apoptosis. Wide variation in the expression pattern was observed for immune-response genes such as PAMP receptors and porcine β-defensins (*PBD-1*, *PBD-2*). Gene expression patterns also vary, even in different intestine regions. Host IRs were observed to be complex, and coworks are usually necessary in resistance to *S. enterica* infections. Based on bioinformatic SNP predictions, SNPs in *HP*, *NCF2*, and *PGD* were associated with *Salmonella* shedding. SNPs were identified in GBP family genes that differentially expressed under *Salmonella* infection. SNPs were observed to be associated with blood parameter traits. It was revealed that the loci controlling the F4ab/F4ac receptor are located on SSC13q41.

In response to *E. coli* infection, mutations observed in functional genes such as HEG1, ITGB5, MUC4, FUT1, B3GNT5, MUC20, *and* MUC13 were reported and found to be associated with porcine susceptibility. In five candidate genes, 34 SNPs within the ETEC F4ab/ac candidate region were identified, but no obvious causative mutations were identified for *E. coli* F4ab/F4ac susceptibility. In the *TF* genome sequence on a panel of 10 different pig breeds, 62 polymorphisms were identified, but a single possible association of the severity of *A. pleuropneumoniae* infection with TF genotypes was observed. Differential gene expression pattern was observed for the *Cav1* gene under *H. parasuis* infection. SNPs in this gene were found to be associated with blood parameter traits.

After candidate gene identification, definite strategies need to be formulated. *First*, identification of candidate genes needs to be strengthened by employing high-throughput approaches, bioinformatics, and functional

genomics. In response to pathogenic agents, different kinds of host IRs would be induced or inhibited simultaneously. Big data analysis is a challenge in attempting to interpret tens of thousands of bits of data and later identifying the right genes against diseases.

Second, candidate genes resistant to multiple pathogens are more useful. In most cases, multiple pathogens play roles simultaneously in porcine infectious diseases—for example, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, and *S. aureus* in porcine lung infections; the presence of pathogenic PRRSV accelerates *H. parasuis* infection.

Third, animal infection models are not limited in pigs. Because some gram-negative bacilli have many hosts, host functional genes in other species may be equally good as references for pig research. Cell cycle and differentiation genes in mouse intestinal crypt epithelial cells following *L. intracellularis* infection were found to be important. In brief, the candidate gene approach has been widely used to identify genes, but very limited mutations have been found to be powerful in breeding schemes. A number of mutations need to be tested in a large field data set. Recent techniques such as high-throughput strategies (e.g., SNPchip and deep sequencing), exome sequencing, bioinformatics, and meta-analysis enable researchers to identify genes in a more efficient way (Tables 19.4 and 19.5).

TABLE 19.4 Candidate genes/pathways against porcine gram-negative bacilli.

S.No.	Causal bacterium	Tissue/organ	Suggested candidate gene/pathway
1	*S. typhimurium*		HP, NCF2, PGD, CCT7
2		Intestine (jejunum, ileum and colon)	*TLR2*, *NOD-1*, *NOD-2*, *PBD-2*, *NF-κB1*, *caspase-1* Regional differences in gene expression profiles and inflammatory response to *S. typhimurium* infection along the porcine intestinal gut exist
3		Macrophage	Enhanced uptake of *S. typhimurium* in macrophages is associated with ERK1/2 activation
4		Intestinal epithelial cell	PBD-1, PBD-2
5		In vivo gut loop model	NOD-2, TLR2, TLR4, TLR5, CCR9, CCRL1
6		Mesenteric lymph node	T Helper 1, innate/inflammatory, and antigen-processing pathways are induced; apoptosis and antigen presentation/dendritic cell function pathways are downregulated; NF-kappa B suppression in antigen-presenting cells may be the mechanism for S. Typhimurium evasion. CD47, CXCL10, SCARB2, INDO, IRF1, SOCS1, STAT1, *SLC11A1*
7	*S. choleraesuis*		14 different chromosomal regions in the porcine genome are found to be significantly associated with susceptibility
8		Mesenteric lymph node	Th1, innate immune/inflammation response, apoptosis pathway, and strong NF-kappa B-dependent response are induced. ARPC2, CCT7, HSPH1, LCP1, PTMA, SDCBP, VCP, INDO, SOCS1, STAT1, SLC11A1
9		Lung	TGM1, TGM3, GBP1, GBP2, C1S, C1R, MHC2TA, PSMB8, TAP1, TAP2
10			Apoptotic pathways, Th1 immune response, and interferon gamma (IFNG) signal are observed
11	*E. coli*		HEG1, ITGB5; MUC4; FUT1; B3GNT5; MUC20; MUC13[18; TFRC; B3GALT3, B4GALT4
12		Duodenum	Genes related to the Glycan Biosynthesis and Metabolism are observed
13		Jejunal mucosa	THO complex 4
14	*A. pleuropneumoniae*	Lung	*MMP-9, MMP-12, TF*
15		Liver	Liver plays an important role in initiating and orchestrating the innate immune response to *A. pleuropneumoniae* infection
16		Peripheral blood leukocytes	*OAS1, CD97, S100A8, TGM3*

Genetic analysis including bioinformatics SNP (single-nucleotide polymorphism) prediction analysis, SNP and association analysis with traits, GWASs (genome-wide association studies), and gene function analysis.

TABLE 19.5 Genes involved in Salmonellosis infection.

S.No.	Gene and abbreviation	Remarks
1	Major histocompatibility complex class I (MHC class I)	Observed to be associated with Salmonella colonization.
2	Major histocompatibility complex class II (MHC class II)	
3	Interleukin 6 (IL6)	Upregulation in resistant chicken
4	Interleukin 8 (IL8)	Upregulation in resistant chicken
5	Interleukin 18 (IL18)	Upregulation in resistant chicken
6	Caspase 1 genes (CASP1)	
7	Natural resistance-associated macrophage protein 1 (NRAMP1)	It is involved in phagocytosis for the reduction of *salmonella enteritidis*
8	Inducible nitric oxide synthase (iNOS)	
9	Complement (C)	
10	Toll-like receptor 4 (TLR4)	
11	Transforming growth factor B4 (TGF-β4)	Downregulation in resistant chick
12	Interferon gamma (IFN-γ)	Downregulation in susceptible chicken IFNγ thus represents a factor to consider in the development of prophylactic measures for the reduction of *Salmonella* carrier state
13	Interleukin-2 (IL2)	Upregulation in resistant chicken small intestine
14	Transforming growth factor B3 (TGF-β3)	
15	TRAIL	
16	Prosaposin	
17	Inhibitor of apoptosis protein 1 (IAP1)	
18	Immunoglobulin light chain (IGL) and	
19	Transforming growth factors B2, B3, and B4 (TGFB2, TGFB3, and TGFB4)	
20	ZOV3	
21	SLC11A1	
22	SAL1	A quantitative trait locus on chromosome 5 was identified that was involved in controlling bacterial load in spleen
23	Myeloid differentiation primary response gene 88 (MyD88)	SNP1 and SNP3 were observed to have significant effect against Salmonella.
24	CD28	
25	Tumor necrosis factor (TNF)	Has a role in Salmonella enteritidis infection
26	MD2	Has a role in Salmonella enteritidis infection
27	Macrophage migration inhibitory factor	Has a role in Salmonella enteritidis infection
28	Tumor necrosis factor alpha (TNF-α factor)	Has a role in Salmonella enteritidis infection

Disease resistance for poultry

Poultry plays an important role in nutritional and socioeconomic security in the livelihoods of poor rural households, women in particular, in developing countries. Scavenging is an important phenomenon in backyard poultry production, which is an integrated part of smallholder production systems. These offer the role of rearing poultry in a zero-input system, which plays a significant role in poverty alleviation. Indigenous chicken varieties were observed to be well adapted to local environments, be excellent foragers, be better able to avoid predator attacks, and demonstrate better immunity to common diseases. It is very important to have genetic resistance against

infectious diseases. A study conducted on indigenous poultry in scavenging condition reported better disease resistance compared with RIR breeds or their crosses. An indigenous chicken breed such as Haringhata Black was observed to be more immune in terms of B cell immunity.

Disease resistance research has included measurement of genetic control of disease losses, estimation of heritability, and characterization of breed or strain differences. Genetic control of most diseases may be the result of the presence or absence of receptors that are simply inherited. Resistance to specific subgroups of leukosis virus in chickens seems to be simply inherited and may be the result of not having the receptor for the virus.

Selection for disease resistance using direct approaches

Direct selection is more effective when a greater number of traits are assessed. Selection with the direct approach includes challenge study with the infective agent. For the selection to become more effective, more than 20 traits are utilized. The basic principle of selection would be to challenge the animal artificially. The genetically resistant animal would represent no symptom of disease. The next step is to select animals accordingly. One of the major constraints is that challenging breeding stock, progeny, or sibs would be costly, depending on the severity of the disease challenge, and accordingly production could be adversely affected. The limitation associated with this technique implies that it is not possible to have a large database of progeny or sibs, because it is not practically feasible to challenge a large number of animals. Cost, ethical concerns, and depressed production are the serious concerns.

Another alternative is to clone the animal, and one set of these animals (clones) could be challenged with a specific disease or diseases. Selection of clones (other animals) could then occur on the basis of results from cloned animals. Because the animals tested are clones, accuracy would be equal to testing the individuals themselves.

Selection for disease resistance using indirect approach

a. Immune response

Immune responsiveness is considered an indirect indicator of disease resistance. Genetic control of antibody response to sheep red blood cells is observed to be moderately heritable. It is an encouraging result that selection for humoral immune response for one antigen may improve humoral immune response for other antigens.

Genetic control of innate and humoral immunity is governed by a series of genes, as discussed in Chapters 5 and 6.

Results suggested that immunization procedures, dosage, and site of immunization all affect the measurement and extent of genetic control. Such details may make the use of immune response as an indicator of disease resistance more difficult. Other experiments have demonstrated that response to vaccination with Newcastle disease, *Salmonella pullorum*, and *E. coli* are under moderate genetic control and that selection for high and low antibody response following vaccination is effective. We had studied that indigenous chicken such as Haringhata Black was observed to have better B cell-mediated immunity compared with that of other poultry species.

b. In vitro methods

A second indirect approach would be to consider in vitro methods as indicators of disease resistance. Phagocytic and bactericidal actions of peripheral blood monocytes act against disease agents such as *Salmonella typhimurium* and *S. aureus*. Other methods include neutrophil metabolic and phagocytic activity and lymphocyte blastogenesis in response to antigens. Lymphocyte proliferation index and neutrophil assay after the use of mitogens as an indicator of cell-mediated response have revealed that genetic differences exist in poultry for the T cell mitogens phytohemagglutinin and concanavalin.

Indirect approaches to selection for genetic resistance to diseases may be summarized as

1. Vaccine challenge

2. In vitro tests

3. Genetic markers

Molecular genetics approaches may consist of constructing disease-resistant genotypes.

c. Marker-assisted selection

The genetic or molecular marker associated with disease resistance may serve as a useful tool for selection of disease-resistant animals. Chapter 7 discusses a set of immune-response genes responsible for disease resistance. Identification of genetic variation at the nucleotide level (revealed through SNPs, CNV, and InDel mutations) and their functional association with the disease of interest will reveal the markers associated with the resistance. MHC class I, II, and III genes may serve as useful markers for disease resistance. The B complex, the MHC in

chickens, has been extensively studied and shown to be involved with both immune response and disease resistance. More specifically, the B complex has been shown to be associated with immune response to synthetic antigens, bovine serum albumen, *Salmonella pullorum* bacterium, total IgG levels, and cell-mediated responses. Resistance to Marek's disease, Rous Sarcoma virus, fowl cholera, and lymphoid leukosis viruses has also been demonstrated to be associated with chicken MHC. More recent molecular genetic approaches have included using gene maps and QTL scans to find genes associated with disease resistance and immune response.

MHC has been linked to resistance to Marek's disease and fowl cholera of chicken. Other genes influencing disease resistance in livestock include the *TNC* gene and the *Ity* gene related to resistance to salmonellosis in chickens. Resistance of chicken against *E. tenella* is due to *MHC-associated* and *non-MHC associated* genes.

Disease resistance for chicken-A case study

Selective breeding has proved to be an efficient system for improving genetic resistance in low-input poultry production systems. It is efficient as a low-cost opportunity for disease control. ILRI has undertaken several projects for the distribution of improved local poultry breeds with improved productivity and production traits as well as better genetic resistance to important infectious diseases such as Newcastle disease, fowl typhoid and fowl cholera, coccidiosis, Marek's disease and IBDV, and ectoparasites and hemoparasites. Genetic resistance to major pathogens for poultry may serve as a key to having a direct application to developed countries. Parasitic and bacterial infections in free-range poultry rearing and organic poultry production are an increasing challenge, and as a result, resistant breeds of poultry may provide the key to disease control. A new program that explores the genes crucial for breeding chickens that can tolerate hot climates and resist infectious diseases (Newcastle disease) has been launched under the leadership of the University of California.

Chicken has 38 autosomes, of which many are relatively small, but uniform in size, known as microchromosomes. Recent prospects of immunogenomics include QTL, mapping of the combination of SNPs, CNVs, indel variations, host immunity, transcriptome analysis, next-generation sequencing strategies, mitochondrial genetics, gene editing, transgenesis techniques for identification of disease-resistant genes, and future application of these techniques for the production of a disease-resistant stock of chicken. Disease-resistant genes for poultry refer to the immune-response genes conferring innate immunity, those coding for antibodies or B cell, microRNA, or others that help the host to resist damage caused by pathogens. Various immune-response genes for poultry have been identified such as NRAMP1, MHC, IFN (interferon) genes, Mx (Myxovirus-resistance) genes, anti-ALV (avian leucosis virus) genes, TLR4, and the Zyxin gene for chicken. We have identified and characterized TLR2, TLR4, TLR7, and Bu-1 genes in chickens and ducks and TLR2 in guinea fowl from our laboratory. The genetic variations may be exploited to study the disease-resistance levels in different organisms. With recent advances in technology and the cost effectiveness of genotyping, the genomic selection approach was observed to be most promising for animal breeding. Chicken genome analysis, development of the chicken transcriptome, and proteome analysis have helped us better understand the disease genetics of chicken and its exploitation for the development of a better disease-resistant stock. Enhanced immune response for chickens has the promise of improving disease resistance or improving efficiency of vaccination. These ultimately reduce the drug residues in food. Currently, the use of antibiotics has been banned in the poultry industry with the aim to reduce drug residues that cause evolvement of antibiotic-resistant pathogens. Some common diseases causing serious economic losses to the poultry industry, with high morbidity and mortality, are discussed as follows:

1. Salmonellosis

Salmonellosis is considered an important disease of poultry causing huge economic losses in terms of mortality, reduced growth, and loss of egg production.

Etiology: Two important species of Salmonella are *S. enterica* and *S. bongori*. *S. gallinarum* and *S. pullorum* are important with great clinical manifestations. Infection may occur at any time over the lifetime of the chicken. Newly hatched chicks are highly sensitive, so infections within the first hours and days of their lives are the most important. Clinical symptoms include lack of appetite, depression, respiratory distress, caseous core diarrhea, and ultimately death in young birds. Symptoms in layers include depressed egg production, fertility, and hatchability.

Prophylactic measures such as vaccination and the use of antibiotics are not sufficient to control the disease. Antibiotic resistance is a major problem encountered, as discussed. Development of a disease-resistant stock is the

most promising field. Attempts are conducted with recent molecular techniques. Identification of direct or indirect markers is a major breakthrough in this path.

Identification of genes involved in salmonellosis:

1. Some of the major genes identified were MHC, caspase 1 genes, NRAMP1, inducible nitric oxide synthase (iNOS), complement, and TLR4.
2. MHC class I was observed to be associated with *Salmonella* colonization. MHC classes I and II were reported to be linked to resistance against *Salmonella* and antibiotic-response kinetics. MHC B haplotypes were observed to be linked with *Salmonella*-specific antibody responses in Vietnam.
3. Differential mRNA expression profile was observed for IL6, IL8, and IL18 with respect to resistant and susceptible chickens. In the mRNA expression in the heterophils of resistant and susceptible chickens, it was shown that the mRNA level of different interleukins such as IL6, IL8, and IL18 were upregulated in resistant chickens compared with susceptible chickens.
4. The mRNA levels of transforming growth factor (TGFβ4) were found to be downregulated in the heterophils of resistant chickens.
5. mRNA levels of interferon gamma (IFN γ) were found to be downregulated in susceptible chickens compared with resistant chickens.
6. Resistance to *Salmonella* has been linked to different genes such as ILs, IFNγ, TLRs, iNOS, and genes involved in apoptosis.
7. Resistant chicken lines showed an upregulated expression of interleukins such as IL2, IL6, IL8, and IFNγ in the small intestines compared with susceptible chicken lines. Interferon gamma gene expression was significantly downregulated in susceptible chicks compared with resistant ones. Interferon γ expression level represents a valuable indication of immunodeficiency associated with persistence of *Salmonella* in the chicken digestive tract. Thus, IFNγ represents a factor to consider in the development of prophylactic measures for the reduction of NRAMP1 carrier state.
8. NRAMP1 is a candidate gene associated with salmonella enteritidis (SE)-mediated immune response and is related to the phagocytosis of SE. Studies have shown that the enhancement of host immunity mediated by the upregulation of NRAMP1 mRNA in heterophil granulocytes and spleen might be more obvious and earlier in SE infection-resistant chicks than in susceptible chicks. Different variations in the NRAMP1 gene have been associated with resistance to salmonellosis. Association analysis indicated that A24101991G is significantly associated with chicken salmonellosis resistance.
9. Certain genes such as NRAMP1, TGFβ3, TGFβ4, and TRAIL have been found to be potent candidates for disease resistance against *Salmonella*. The candidate gene approach is a useful method to investigate the genes involved in genetic resistance.
10. Twelve candidate genes were reported in the pathogenesis of *Salmonella* in meat-type chicken. These genes include NRAMP1, prosaposin (PSAP), inhibitor of apoptosis protein 1 (IAP1), inducible nitric oxide production (iNOS), caspase-1 (CASP1), interferon-gamma (IFNγ), immunoglobulin light chain (IGL), interleukin-2 (IL2), transforming growth factors B2, B3 and B4 (TGFB2, B3 and B4) and ZOV3. *Salmonella enteritidis* infection was given to birds at 3 weeks post hatch. At day 7 post infection SE load was quantified in caecum, spleen and liver contents. In caecum nine out of 12 genes were found to be associated with bacterial load. These genes include CASP1, SLC11A1, IAP1, PSAP, iNOS, IL2, TGFB2, TGFB4, and IGL. Five genes (SLC11A1, 1L2, CASP1, IGL and TGFB4) were found to be significantly associated with bacterial load in liver. Only one gene i.e., TGFB3 was found to show association with bacterial load in spleen. The above study confirmed polygenic nature of SE resistance.
11. A QTL on chromosome 5 was identified, involved in controlling bacterial load in spleen and was named as SAL1. This QTL was found to be involved in bacterial clearance by macrophages.
12. SNP studies have shown three SNPs in an exon of chTLR15. One of the SNPs was found to be associated with *Salmonella* infection. The "T" allele in SNP C726T might be linked to resistance of *Salmonella* infection. The mRNA expression of TLR15 in heterophils of chickens infected with SE was downregulated than that of the control group at third day of post infection. However, TLR15 was upregulated in the spleen of chickens infected by SE at day 3 pi. The above discussed genes are potential candidates that can be used for selection programs for increasing genetic resistance against *Salmonella* Enteritidis in chickens.
13. A number of factors that include NRAMP1, MHC, TLR4 and a novel genetic locus SAL1 determine the genetic resistance of chicken against SE.
14. After analysing and comparing studies of MyD88, novel mutation G4810372T was found that was thought to have an effect on immune response of the individual. Further studies are needed to elucidate the molecular

mechanisms that occur due to MyD88 gene polymorphisms. After correlating susceptibility toward *Salmonella* Pullorum and MyD88 polymorphisms, it was found that alleles in SNP1 locus and SNP1 and SNP3 genotypes reported a significant effect against *Salmonella.* Also the advantaged haploid type combined by SNP1, SNP3 and SNP4 loci played a very significant role in genetic resistance to *Salmonella* Pullorum infection. Myeloid differentiation primary response gene 88 polymorphisms or advantaged haploid type in a particular region had a positive effect against susceptibility to *Salmonella* Pullorum infection. From the above observations it can be concluded that MyD88 can be used as a candidate gene which could provide a conceptual reference for MAS for poultry.

15. In a study that was based on the biological function and SE response of various genes, five candidate genes were selected that were found to have a role in *Salmonella enteritidis* infection. These genes include TLR4, macrophage migration inhibitory factor, T cell-specific protein (CD28), TNF-α, and MD-2. In TLR4, CD28, and MD-2, SNPs were found. The SNPs were tested for associations between sire SNP and *Salmonella* enteritidis response. The association of sire SNP with cecum bacterial load and vaccine antibody response was found to be statistically significant. Association of MD2 SNP was statistically significant with bacterial load in spleen. The use of the above studied SNPs can be used in MAS and may result in improvement in diseases resistance in poultry.

2. Avian influenza virus

Avian/bird flu is considered one of the most deadly diseases with extreme zoonotic importance, caused by the avian influenza virus (AIV) belonging to the Orthomyxoviridae family. It is the most fearful viral disease of birds and has the potential to cause a detrimental effect on poultry flocks with huge mortality and major economic losses for the poultry industry. This disease is of great economic and zoonotic importance and may also lead to pandemic threats. The details have been described in Chapter 2.

Genes involved in avian influenza virus—many studies have been carried out to determine different disease-resistant mechanisms and genes in AIV:

1. Studies on Beijing-You chickens have identified 39 SNPs associated with different immunological traits against AIV. An important QTL was observed on chromosome 16 related to total $I_g\gamma$ concentration. Also, five candidate genes related to $I_g\gamma$ levels were revealed that might play a role in the immune modulation of birds infected with AIV. Different candidate SNPs for MAS for disease resistance have been detected. The candidate genes play a vital role in regulating immunological response in chickens.

2. Approaches such RNAi technology have attempted to develop transgenic poultry resistant to AIV. Synthetic RNA duplexes (siRNA) can be employed to trigger RNAi. Also, RNAi can be triggered by the expression of RNA duplexes in hairpin structures (shRNA) that can be processed into siRNA by RNA endonucleases. siRNA specific for conserved domains of the influenza virus genes have been observed to inhibit replication of various influenza viruses in a study on chicken cell lines developed from embryo. Stable expression of influenza-specific shRNA via a lentiviral vector in a cell line makes the cells refractory to influenza virus infection. After introduction of the above-mentioned lentiviral vector into mouse lung, an inhibition in virus production was detected in vivo. Hence, there is a possibility to develop influenza-resistant poultry flocks by transgenic expression of influenza-specific shRNA.

Hence in the current era, avian influenza-resistant chicken can be produced using a combination of transgenesis and RNAi that can be used for AIV gene expression inhibition. Screening of siRNAs as candidate genes in vitro is the key step for transgenic breeding. Bioinformatics along with other online search tools is helpful for designing siRNAs that target different mRNA sites of the AIV H5N1 subtype. Five rational siRNAs were chosen and five U6 promoter-driven shRNA expression plasmids that contained the siRNA genes were constructed and used to develop stably transfected Madin—Darby canine kidney cells.

3. Indirect immunofluorescent antibody, virus titration, PUI stained flow cytometry, RT-PCR, and DAS ELISA revealed that all five stably transfected cell lines, when exposed to $CCID_{50}$ of AIV, were resistant to viral replication. Finally, transgenic chicken were developed from the plasmids (pSi604i and pSi 1597i). These findings provide baseline information for breeding transgenic chickens resistant to AIV in combination with RNAi.

4. Although to date transgenic bird flu-resistant chickens are not available, transgenic chickens have been developed with some success in minimizing the spread of AIV but have been unable to prevent the emergence of disease.

5. In our laboratory, we have undertaken one research project funded by the Science and Engineering Board, Department of Science and technology, Government of India, titled "*Genetic characterization of indigenous ducks and study of its innate immunity particularly against avian influenza.*" To our observation, we have found that duck,

an important poultry species, was asymptomatic to avian influenza. Our main aim is to identify the potential genes present in ducks which confer immunity against avian influenza. In the later part, we can insert the gene in chickens for the development of bird flu-resistant transgenic chicken production. It is an ongoing project, and in the initial phase we have identified RIG1, TLR3, and TLR7 as important potent genes that bind with the AI protein as H (hemagglutinin) and N (neuraminidase) through in silico studies. It is very interesting to note that the chicken genome is devoid of RIG1, whereas it is present and expressed well in ducks.

3. Marek's disease

Marek's disease (MD) is an important disease of chicken caused by the herpes virus as Marek's disease virus (MDV). It is a neoplastic disease of chicken, causing huge economic losses for the chicken industry. MD signs include depression, wasting, loose watery stool, paralysis, lymphomas, and severe immunosuppression. Vaccination programs have been used to control onset of the disease, but MDV still replicates in vaccinated chicks, so this has limitations. These highly contagious cell-free virions are continuously shed in the environment, which makes MDV environmentally persistent as well as highly infectious. Continuously, more virulent MDV strains evolve that make current vaccination programs ineffective and create the urge to develop strategies that will augment existing MDV control strategies.

Genes involved in resistance to Marek's disease—the genetics of host response and host genetics to MDV have been studied for years. Many loci have been known to be involved in disease resistance, but only a few genes have been identified as having an actual role:

1. MHC plays a vital role in resistance against MD. Being a polygenic trait, many genes and gene loci have been reported to be involved in MD resistance. MHC is one among gene/loci to be involved in genetic resistance against MD.
2. Other genes that are non-MHC in origin have also been linked to play a role in genetic resistance/susceptibility to MD. These genes include growth hormone gene, cytokines (IL6 and IL18), and the stem lymphocyte antigen 6 complex (LY6E) gene.
3. Loci rs14527240 and GGaluGA156129 have been reported to play a role in host resistance/susceptibility to MD.
4. Expression studies suggest a possible role of SMOC1 gene in MD susceptibility.
5. Nitric oxide, apart from being a promising antiviral agent, plays a role in modulating immunological responses. While working on MD it was found that chickens resistant to MD have the ability to have more enhanced nitric oxide production than that of susceptible chicken lines. The above observation was made by measuring nitric oxide levels from chicken fibroblasts taken from these chicken lines after treatment with LPS and recombinant chicken IFN-γ. Further plasma nitric oxide levels were measured in chicken lines (N2a, P2a) inoculated with JM-16 strain of MDV. The levels of NO were found to be increased in N2a chickens in the majority of experiments carried out. The level of the NO production was found to be associated with the range of virulence of the MDV strain. Inoculation with more virulent strains induced the highest NO levels, which suggests a possible role of NO during disease progression.
6. Quantitative RT-PCR studies depict that IFNγ does not primarily induce iNOS gene expression during MDV infection. Nitric acid production and inducible nitric oxide gene expression are mediated during the cytolytic phase of infection. These findings suggest that NO may play a role in increasing MDV virulence by suppressing the immune system.
7. In order to breed chicken genetically resistant to MD, it is essential to have sufficient knowledge about markers that play a role in resistance to MD. A study was carried to identify the MD-resistant markers in chicken lines. Copy number variation (CNV) was studied in inbred MD-resistant and susceptible chicken lines. In four chicken lines, 45 CNVs were detected, of which 28 were involved in cellular proliferation and immunological responses. Also, two CNVs observed to be associated with resistance to MD were inherited to the descendant recombinant congenic lines that differ in MD susceptibility. These observations may be useful for designing better and more reliable strategies to improve genetic disease resistance in poultry.

4. Newcastle disease

Newcastle disease, commonly known as ranikhet disease, had a high prevalence causing heavy mortality. The causative agent of Newcastle disease is Newcastle disease virus (NDV), which belongs to paramyxovirus and is a negative-sense RNA consisting of about 15×10^3 nucleotides. This is an enormously destructive and contagious disease that causes serious problems in the poultry industry globally. Among different poultry diseases, NDV was reported to be the fourth-most-destructive disease and led to heavy losses for the poultry industry. Newcastle disease is considered the most widespread disease in animals along with rabies and bovine tuberculosis. Nonspecific symptoms for NDV include ruffled feathers, depression, breathing problems, anorexia, hyperthermia, and listlessness followed by death. Affected chickens show respiratory and neurological

complications and also a reduction in egg production. Chickens infected with NDV are able to raise an antibody and gene response. The antibody response varies in different chicken breeds; hence understanding the genetics of the immune response may help in improving disease resistance in chickens.

Genes involved in resistance to Newcastle disease—a study was conducted to elucidate the host antibody response to NDV:

1. A novel QTL found to be associated with antibody response was identified. From the proximal end of GGA1, this QTL region was located approximately 100 Mb away. This region was predicted to play an important role in the immune response of the chicken. Two genes, namely ROBO1 and ROBO2, were observed to be promising candidate genes that might have a role in modulating antibody response in chickens infected with NDV. For further confirmation of the role of these genes, silencing and overexpression of ROBO1 and ROBO2 need to be carried out both in vivo and in vitro. Host response toward NDV infection is poorly understood.

2. A transcriptional profiling study of chicken embryo cells was carried out in order to have a better understanding of host—pathogen interactions during NDV infection. Analysis of chicken embryo cells infected with NDV strain D58 was carried out by quantitative RT-PCR. Some genes under study were upregulated and some were downregulated.

Genes such as IFN-α, IFN-γ, DDX-1, and MHC-1 were upregulated, while the IL6 gene was downregulated. Expression levels of the M and F genes of the virus were also measured. The genes that encode for proinflammatory response, cellular responses, and other genes that regulate interferons were found to be affected during the infection. The observations suggest the involvement of different signaling pathways that are involved in host response toward infection.

A case study

DNA region in chickens identified for disease resistance

One region in the DNA was claimed to explain a large difference in possible disease resistance between chickens. This was discovered by researchers of Wageningen University and Research and Hendrix Genetics. This DNA region contains, among others, an *important sensor* for activating the immune system, which might explain the fact that some chickens become diseased while others remain healthy. This discovery may form a new path for genomic selection of disease-free chickens. As a consequence, disease-free stock may be developed so that the use of antibiotics is minimized to zero.

Identification of DNA region

To better understand which DNA region(s) contribute to heritable variation, researchers of Wageningen University and Research and Hendrix Genetics investigated the whole genome of more than 1600 chickens. Tom Berghof was the lead researcher of this study. They used genetic differences in the whole genome to identify DNA regions that occur more often in chickens with high natural antibody (NAb) levels or in chickens with low NAb levels. One region was detected with a very large effect on NAb level, which explained more than 60% of the genetic variation observed. Within this region, one candidate gene was eventually identified. The region was observed to contain a few genes. It is very difficult to identify the difference at the DNA level that explains the difference in NAb level. Most likely this difference is due to the *TLR1A* gene, which makes it the main candidate.

Toll-like receptors

TLR1A is member of the TLR family, an important part of the immune system. This is a group of receptors, a type of sensor, that recognizes common structures on pathogens. These proteins detect certain parts present on many bacteria or viruses. These sensors therefore have a very broad function. In humans, genetic mutation in TLRs have been associated with increased or reduced risk for diseases. Based on the study of Berghof and colleagues, this seems to be the case as well in layer chickens. The association with NAb was claimed to be new, independent of the species.

Natural antibodies

Antibodies are proteins produced in diseased animals that are triggered by antigens through the mechanism of humoral immune response. The details have been discussed in Chapter 6. These antibodies are only made after the animal has become infected with the pathogen. However, in addition, an animal also has **NAbs**. NAbs have only recently been discovered in livestock. These antibodies are already present in healthy animals without any previous exposure to a pathogen. NAbs are important for fighting pathogens—they inhibit and prevent further infection in the body. Simultaneously, they also warn and activate other parts of the immune system. Earlier studies show promising results. Layer chickens with higher NAb levels were observed to be associated with better chances of survival. NAb levels were observed to be heritable and thus can be influenced by breeding and can be employed for improvement programs against diseases.

Applications and future plans

This study offers direct applications for breeders to select layer chickens for increased disease resistance by selecting for this specific DNA region. Currently investigations are ongoing for the application of these research findings into the breeding programs of a purebred line. Three field experiments with layers with high or low NAb levels are in progress. These hens will be monitored for livability and production. Plans have been made to investigate the *TLR1A*-sensor. Future research is needed to investigate the role of *TLR1A* in the immune system of chickens. In the long term, this could result in improved vaccines and health-promoting nutrition. Eventually this should lead to animals with a higher general disease resistance with lower antibiotic use, lower costs for farmers, and higher animal welfare.

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